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Synthesis of Pyridinium Amphiphiles Used for Transfection and Some Characteristics of Amphiphile/DNA Complex Formation

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Pyridinium amphiphiles have found practical use for the delivery of DNA into cells. Starting from 4-methylpyridine, a general synthesis has been devised for the production of pyridinium amphiphiles which allows variation in both the hydrophobic part and in the headgroup area of the compounds.

By means of differential scanning microcalorimetry, zeta potential, particle size measurements and cryo electron microscopy, some characteristics of the pyridinium amphiphile/DNA complexes have been determined.

Introduction

In recent times cationic amphiphiles have established a firm position as reliable (re)agents for the delivery of DNA, RNA, and oligonucleotides into eukaryotic cells. Although the mechanism of action is not yet clear, in several structure–activity studies an appreciable number of cationic amphiphiles which promote the delivery of exogenous DNA into mammalian cells have been reported.^[1] In addition, with respect to the *in vivo* delivery of polynucleotides, cationic amphiphiles are regarded as a viable alternative to viral-based methods.^[2] The latter are associated with risks attendant with the uncontrolled recombination of viral DNA with the host DNA, and the immunological reaction generally related to the use of viruses. With the use of cationic amphiphiles these risks are avoided, although their use *in vitro* is generally accompanied by cytotoxicity to a variable extent. Compared to viral-mediated gene delivery, the cationic amphiphiles are simple to use and display much greater versatility in the type of material which can be delivered. Ultimately these developments may lead to acceptable protocols for gene therapy.

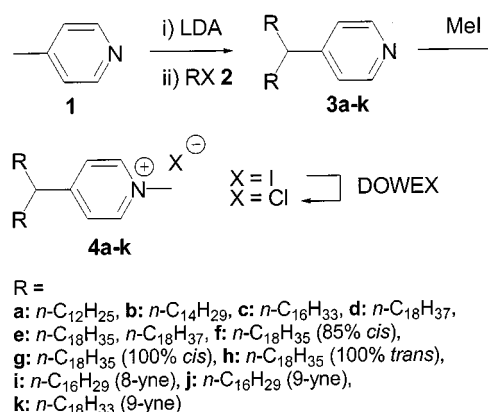
In general, mixing of the cationic amphiphile with the polynucleotide of choice is sufficient for the formation of the active complex or so-called lipoplex.^[3] Previously, we have reported on the capability of pyridinium amphiphiles,

called SAINT (Synthetic Amphiphiles INTERdisciplinary), to transfect efficiently eukaryotic cells.^[4] Here we describe in detail the synthesis of these pyridinium amphiphiles. In addition, we report some physiochemical properties of the SAINT amphiphiles and amphiphile/DNA complexes which may be relevant for influencing the efficiency by which these amphiphiles deliver DNA into the cell.

Results and Discussion

Synthesis

The alkylpyridinium amphiphiles were synthesized by double alkylation of 4-methylpyridine (**1**) by deprotonation with 2 equivalents of LDA followed by the addition of 2 equivalents of the appropriate alkyl bromide or alkyl iodide **2** (Scheme 1). For the preparation of **3e**, two separate alkylation steps were carried out successively. By using this general procedure a representative series of 4-alkylated pyridines **3** were synthesized. Quaternisation of the nitrogen of the resulting alkylated pyridine with methyl iodide and



Scheme 1. Synthesis of SAINT amphiphiles

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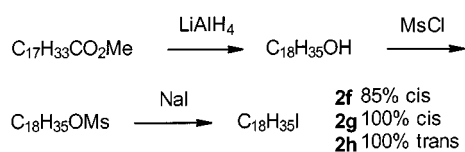
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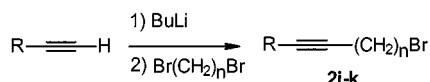
subsequent ion-exchange gave the desired pyridinium amphiphiles **4** as the chloride salts.

Whilst the saturated alkyl halides **2a–2d** are commercially available, unsaturated alkyl halides had to be prepared. Technical grade oleyl alcohol, 85% *cis*-isomer, was used for the preparation of oleyl iodide. Mesylation of the alcohol, followed by reaction with sodium iodide yielded the desired iodide **2f**. For the preparation of the alkyl halides having either 100% *cis* (**2g**) or *trans* (**2h**) orientation, the corresponding commercially available methyl esters were used as precursors. Reduction with lithium aluminium hydride afforded the corresponding alcohols, which were then converted into the desired iodides as described above (Scheme 2). The ratio of *cis* to *trans* double bond in the final products was determined by double resonance NMR experiments.



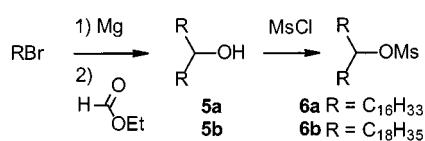
Scheme 2. Synthesis of alkenyl iodides

For the preparation of internal alkynes a common strategy based on bromination of internal alkenes followed by di-dehydrobromination proved troublesome. Alternatively, the desired alkynyl bromides **2i**, **2j**, and **2k** were synthesized by deprotonation of terminal alkynes followed by alkylation with an excess of an α,ω -dibromo alkane (Scheme 3). This approach has the advantage that long-chain alkene bromides with the double bond at different positions may be prepared by partial hydrogenation of the internal triple bond.



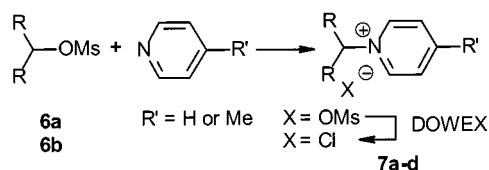
Scheme 3. Synthesis of alkynyl bromides

In order to investigate if the position of the positive charge in the pyridinium ring has an influence on the transfection efficiency, amphiphiles with an “inverted headgroup” were synthesized. For the preparation of the required amphiphiles, a straightforward approach (which anticipated direct alkylation of the pyridine nitrogen with the hydrophobic moiety of interest) was adopted. The compounds **6**, which contain alkyl chains with a leaving group at the central carbon atom, were obtained by a double Grignard reaction with ethyl formate,^[5] followed by mesylation of the resulting alcohol **5** (Scheme 4).



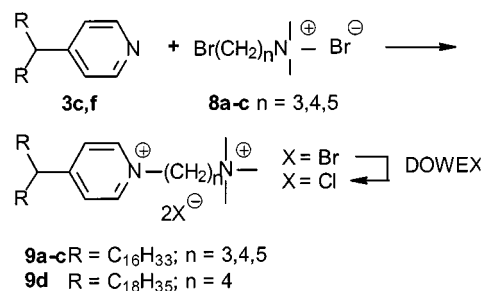
Scheme 4. Synthesis of double-chained mesylates

For satisfactory yields of the desired cationic amphiphiles **7**, the alkylation step was performed in neat pyridine or 4-methyl pyridine (Scheme 5).



Scheme 5. Synthesis of SAINT amphiphiles with inverted headgroups

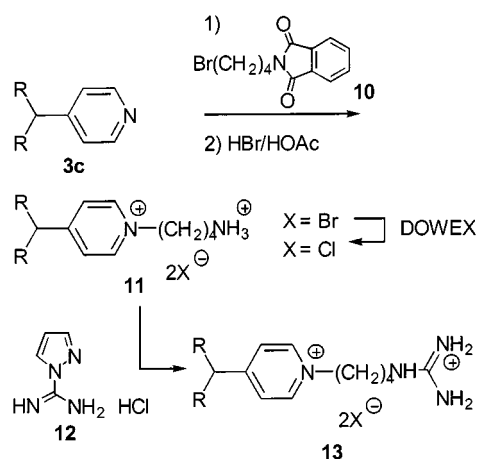
Besides variation in the hydrophobic part of the amphiphile, variation in the headgroup was also pursued in attempts to improve the efficiency of transfection. In particular, introduction of additional cationic charge could result in a more profitable interaction with the DNA to be delivered into the cells. As described above for the “inverted headgroup” pyridinium amphiphiles, pyridines can be readily alkylated on the nitrogen with a variety of alkyl halides. Quaternary amines are the cationic functionality of choice to be incorporated into the headgroup. A series of bromoalkyltrimethylammonium bromides **8** with different (short) alkyl chain lengths was synthesized following a known procedure.^[6] Reaction of a dibromoalkane in ether with a solution of trimethylamine in ethanol results in substitution of one bromine by a trimethylamine group with precipitation of the desired product. Disubstitution is thus prevented. Alkylation of the 4-substituted pyridines in refluxing ethanol with the bromoalkyltrimethylammonium salts results in amphiphiles having two positive charges separated by a short spacer (**9**) (Scheme 6). A twofold excess of a 4-substituted pyridine, which, after complete conversion of the bromide could be easily recovered by chromatography, was used in these reactions.



Scheme 6. Synthesis of double-charged SAINT amphiphiles

A primary amino group was introduced by a slightly different route. The alkylating agent 4-bromobutyl-phthalimide **10** (commercially available or readily prepared from 1,4-dibromobutane and potassium phthalimide) was used. Again an excess of 4-substituted pyridine was employed in order to allow the reaction to proceed at an appreciable rate and to give a satisfactory yield. It was observed that after chromatography over Al_2O_3 (neutral, activity III) some of the phthalimide product was hydrolysed to leave an acylated amino group. Treatment of the resulting mixture of compounds with hydrogen bromide in acetic acid afforded the

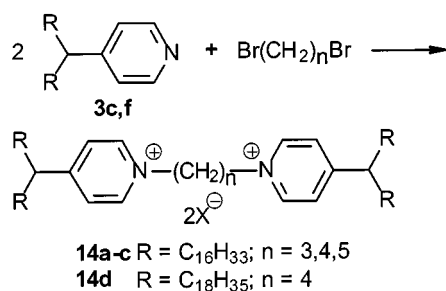
pyridinium amphiphile bearing a free amino group **11** (Scheme 7).



Scheme 7. Synthesis of ammonium and guanidinium substituted pyridinium salts

The amino group in amphiphile **11** was converted into a guanidinium group with **12** (prepared from pyrazole and cyanamide^[7]).

Another class of interesting amphiphiles are the so-called gemini compounds **14** in which the headgroups of two amphiphiles are linked via a carbon-chain spacer of variable length. In order to achieve disubstitution of α,ω -dibromo alkanes by **3c, f** more than two equivalents of pyridine are required and prolonged reaction times are necessary to achieve complete disubstitution. It is important here not to employ too high reaction temperatures to avoid degradation of the 4-substituted pyridines. Acetone is the solvent of choice (Scheme 8).



Scheme 8. Synthesis of gemini SAINT amphiphiles

Some Characteristics for Amphiphile/DNA Complex Formation

As evidenced by transmission electron microscopy, all amphiphiles used in this study form bilayer structures (data not shown). In the complex interactions between such vesicles and DNA, the fluidity of the bilayer plays an important role. It should be noted that *in vitro* transfections on cell-cultures are normally performed at 37 °C. For the compounds **4a–d** the main phase transition temperatures were determined using differential scanning microcalorimetry. This was carried out for the pure compounds as well as for mixtures of the pyridinium amphiphiles with dioleoylphosphatidylethanolamine (DOPE), a phospholipid commonly used as co- or helper-lipid in transfections employing ves-

icles of cationic amphiphiles. It has been suggested that DOPE plays a role in destabilising the target membrane through which the DNA has to be delivered.^[8] For transfection studies *in vivo*, vesicles are generally prepared from mixtures of cationic amphiphiles and cholesterol. The cholesterol serves to stabilise the bilayer, consequently resulting in a longer circulation time. We have determined the phase transition temperatures of 1:1 mixtures of cholesterol and the pyridinium amphiphiles and the data presented in Figure 1.

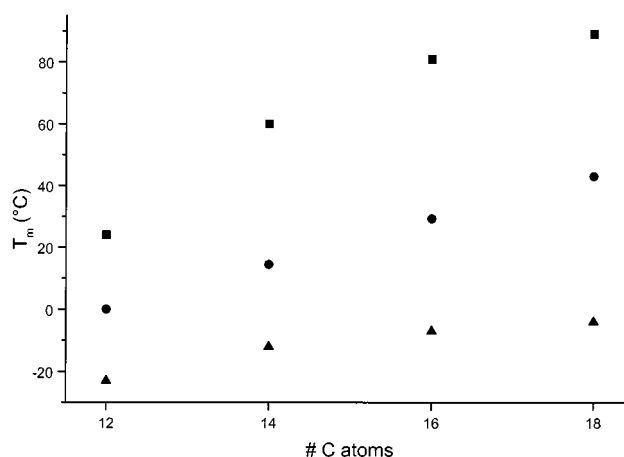


Figure 1. Phase transition temperatures of amphiphiles **4a–d** pure (circles), as a 1:1 mixture with cholesterol (squares) and as a 1:1 mixture with DOPE (triangles)

As expected, a linear relationship between T_m and the chain length is observed for the pure compounds. Previously it has been shown that in mixtures there is a deviation of this linearity.^[9] There is a substantial influence on the stability of the bilayers by DOPE as well as cholesterol. All amphiphile/DOPE bilayers are in a liquid crystalline phase at the temperature at which transfections are performed and in the presence of cholesterol packing in the bilayer is much tighter. Whether or not this has (a) an influence on the time the complex of DNA and cationic amphiphile/cholesterol will circulate in an organism and (b) an influence on the efficiency of DNA delivery has yet to be established. Interestingly, *in vitro* the cholesterol-containing complexes transfect cells as effectively as DOPE-containing complexes, although compared to the latter a two-fold higher concentration is usually required to obtain the same transfection efficiency (unpublished observations). Finally we note that amphiphiles with unsaturation in the chain have phase transition temperatures below 0 °C.

The overall charge of the active amphiphile/DNA complex is of importance for the efficiency of cationic delivery systems.^[10] Apart from interaction with the negatively charged phosphate ester moieties of the DNA backbone, a surplus of positive charge is needed for interaction with the net negatively charged cell membranes. By determining the electrokinetic potential (zeta potential), a measure for the net positive charge of the amphiphile/DNA complex can be obtained. These zeta potentials were determined for a series of complexes of **4f**/DOPE (1:1) vesicles and DNA. The DNA concentration was kept constant while the concentra-

tion of **4f**/DOPE vesicles was varied. With equimolar amounts of both DNA and **4f**/DOPE vesicles, a strongly negative zeta potential, of ca. -20 mV, was measured. This value corresponds with those obtained when there is an excess of DNA. From approximately a $+/-$ charge ratio of 1.5 the zeta potential turned strongly positive, ca. 35 mV, and remained at this value up to a $+/-$ ratio of 7. Optimal transfection is found at a $+/-$ charge ratio of 2.5.^[4b] Interestingly, the “inverted headgroup” amphiphile **7d** gives similar values. Obviously the value of the zeta potential is not a

dominating factor in determining whether a complex will transfect a cell efficiently. These data do suggest however, that the bilayer remains intact on complex formation. At equivalent charge concentration not all negative charge is neutralized, resulting in an overall negatively charged complex. However, at less than 2 equivalents of positive charge, the bilayer, while remaining intact, has rearranged thus compacting the DNA.^[11] This notion could not be substantiated by particle size measurements, employing dynamic light scattering, due to the polydispersity of the samples. Typically particles of 120 to 220 nm in diameter were observed. At $+/-$ charge ratios greater than 5, larger aggregates, 600–800 nm, were formed.

Direct information on the structure of the amphiphile/DNA complex was obtained by cryo-electron microscopy (EM). Vesicles of **4f**/DOPE 1:1 were prepared by sonication in water and were analysed by cryo-EM. A large population of vesicles of 30–40 nm in size was observed. On addition of plasmid DNA, 4700 base pairs in size, resulting in a $+/-$ charge ratio of 2.5, aggregates of approximately 150–200 nm in diameter were found. All vesicles appeared to be recruited by the DNA, and no “empty” or small vesicles could be observed. Extruded vesicles of 100 nm size, as shown in Figure 2a, were also prepared. When DNA was added, only the dense structures (represented in Figure 2b, c) were found. Again a rather uniform aggregate size was observed. In some areas spikes, protruding at the surface of the aggregates, can clearly be seen (Figure 2d arrows). It would be of interest to investigate to what extent the size of the DNA dictates the size of its aggregate with cationic amphiphiles. The amphiphile/DNA aggregates clearly reveal a specific association which is tentatively recognized as a bilayer sandwich. Lammellar structures were found at a repeat distance of 6.5 nm, similar to structures reported in the literature.^[12]

Transfection Experiments

Transfections were carried out using a β -galactosidase assay.^[13] Briefly, vesicles of cationic amphiphiles and DOPE (1:1) were mixed with plasmid DNA which contained an encoded reporter protein. The resulting aggregate was put onto cultured cells (COS-7) and after 2 days the cells were analysed for the presence of the reporter protein. A selection of the results is listed in Table 1. The transfection efficiencies are given in arbitrary units and compared to the efficiency of Lipofectin [a mixture of dioleoylpropyl trimethylammonium chloride (DOTMA) and DOPE], which is one of the most widely used transfection reagents.^[14]

It is evident from these results that introduction of unsaturation in the alkyl chains increases the transfection efficiency. The orientation, *cis* or *trans*, and in particular, the bond multiplicity i.e. double or triple of the unsaturated group have a significant influence. Furthermore, the introduction of an additional positive charge in the headgroup strongly affects the transfection efficiency, depending upon the nature of the second cationic moiety. For example, an additional trimethylammonium group **9a–9d** has a large decremental effect on the transfection efficiency. The gemini

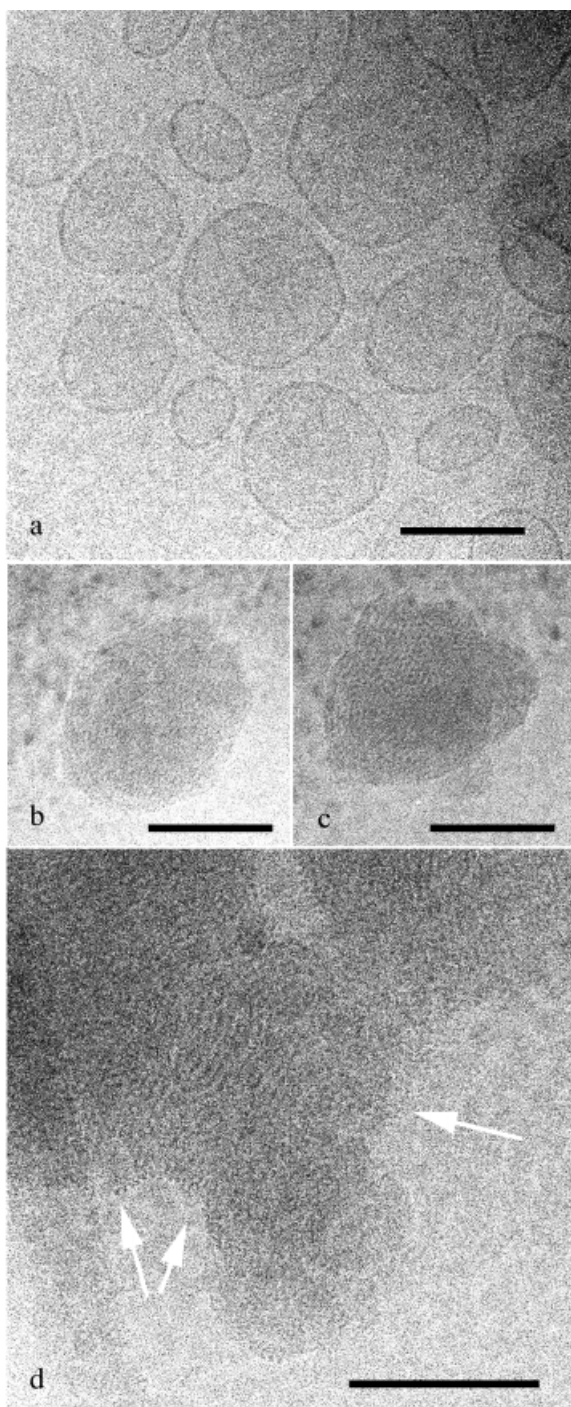


Figure 2. Cryo-TEM micrographs. Top: vesicles of **4f**/DOPE (1:1). Bottom: complex of vesicles of **4f**/DOPE (1:1) with DNA. Bar represents 100 nm

Table 1. Transfection efficiencies (TE) of SAINT amphiphiles, in arbitrary units

Compound	TE
Lipofectin	1
4a	R = C ₁₂ H ₂₅ 0.5
4b	R = C ₁₄ H ₂₉ 1
4c	R = C ₁₆ H ₃₃ 1
4d	R = C ₁₈ H ₃₇ 0.5
4e	R = C ₁₈ H ₃₅ ; R' = C ₁₈ H ₃₇ 1.6
4f	R = C ₁₈ H ₃₅ (85% <i>cis</i>) 4.8
4g	R = C ₁₈ H ₃₅ (100% <i>cis</i>) 5.9
4h	R = C ₁₈ H ₃₅ (100% <i>trans</i>) 2.1
4i	R = C ₁₆ H ₂₉ 1
4k	R = C ₁₈ H ₃₃ 2
7b	R = C ₁₆ H ₃₃ ; R' = CH ₃ 0.1
9a–c	R = C ₁₆ H ₃₃ ; n = 3, 4, 5 0
9d	R = C ₁₈ H ₃₅ ; n = 4 0
14a–c	R = C ₁₆ H ₃₃ ; n = 3, 4, 5 1
14d	R = C ₁₈ H ₃₅ ; n = 4 0.1

SAINTS **14a–14c** have a performance similar to that of lipofectin, but in the case of octadecenyl chains **14a**, the transfection ability is reduced. We note, however, that the effects of unsaturation and additional charge separately are not additive. Interestingly, inversion of the headgroup **7** leads to a large decrease in the ability to deliver DNA.

Conclusions

A number of cationic pyridinium amphiphiles have been synthesized in order to identify structural parameters that influence the efficiency of these amphiphiles to deliver DNA into eukaryotic cells. Unsaturation in the hydrophobic moiety of the amphiphile has the most marked effect. An additional cationic charge in the headgroup also affects the transfection efficiency. Both effects are not synergistic. Mixed bilayers of pyridinium amphiphile and DOPE are in a fluid state under the conditions for transfection. As evidenced by cryo-EM, well-defined SAINT/DNA complexes are formed. Atomic force microscopic studies of the structure of the amphiphile/DNA complexes are currently in progress.

Experimental Section

General: All reactions were carried out in dried solvents under a nitrogen atmosphere using oven-dried glassware. – For column chromatography Al₂O₃ (activity II–III), prepared by adding the indicated amount of water to Merck aluminium oxide 90 active, neutral (activity I), and Merck silica gel 60, 230–400 mesh were used with the indicated eluents. – Melting points (uncorrected) were determined using a Koffler melting point microscope. Several compounds displayed liquid crystalline behaviour, in these cases no melting points are reported. – NMR: Varian Gemini 200, Varian VXR 300. – MS: NERMAG R 3010 triple quadrupole mass spectrometer equipped with an in house built atmospheric pressure ionisation source and ionspray interface. – Elemental analyses: Analytical Department of the University of Groningen, carried out by Mr. J. Ebels, Mr. J. Hommes, and Mr. H. Draaijer. Accurate

elemental analyses were difficult to obtain due to the presence of shorter and longer carbon chain homologues in the alkyl-chain starting materials, as was evidenced by electrospray mass spectrometry. – Differential scanning calorimetry (DSC) measurements were carried out on a Perkin–Elmer DSC-7 apparatus. Sample concentration was 10–40 mM. Scan rate was 1 °C/min. From 5 °C to –5 °C, the scan rate was 0.5 °C/min and at 0 °C an equilibration time of 30 min was employed. – The zeta potential measurements were performed on a MALVERN 200 apparatus, equipped with a He/Ne laser. The concentration of DNA used was 15 mg/mL in 20 mM Hepes buffer, pH 7.4. An average molecular weight for a nucleic acid monomer of 330 was taken to calculate the concentration of negative charge. Dynamic light scattering was performed on a Neyvis instrument equipped with an Ar laser. Threefold diluted samples used for the zeta potential determinations were employed. – Cryo-electron microscopy was performed on holey carbon films. Images were recorded under low dose conditions on a Philips (CM 120) electron microscope operating at 120 kV, equipped with a Gatan model 626 cryo stage. Samples were 2.4 mm in DNA concentration and were prepared in water. Transfections were performed as previously described.^[4]

Synthesis of Oleyl Iodides 2f–h: A solution of mesyl chloride (6.5 mL, 84 mmol) in dichloromethane (50 mL) was added dropwise to an ice-cold solution of oleyl alcohol (20.5 g, 76.3 mmol) and triethylamine (15.5 mL, 1.5 equiv.) in dichloromethane (350 mL). The mixture was stirred at room temperature. The reaction was monitored with NMR spectroscopy and quenched as soon as the oleyl alcohol had disappeared (after 15–60 min) by the addition of water. The organic phase was washed successively with HCl, NaHCO₃, and brine then dried (Na₂SO₄), and the solvent evaporated to give the desired mesylate as a viscous oil (25.5 g, 73.6 mmol, 96%). The precursor for **2f** is a solid at 0 °C. The precursors **2g–h** were crystallized from ethanol: m.p. *cis*-mesylate 9 °C, m.p. *trans*-mesylate 32 °C. – ¹H NMR (200 MHz, CDCl₃): δ = 0.87 (t, 3 H, CH₃), 1.26 (m, 22 H, CH₂), 1.67–1.78 (m, 2 H, CH₂CH₂OMs), 1.97–2.05 (m, 4 H, CH₂CH=CHCH₂), 3.00 (s, 3 H, OMs), 4.20 (t, *J* = 6.6 Hz, 2 H, CH₂OMs), 5.32–5.37 (m, 2 H, CH). Sodium iodide (5.1 g, 34 mmol) was added to a solution of the mesylate (9.26 g, 26.7 mmol) in acetone (100 mL) and the reaction mixture heated under reflux for 2 h. After cooling to room temperature, the mixture was filtered, the filtrate evaporated and then taken up in hexane and again filtered through Al₂O₃. On evaporation of the solvent a clear oil was obtained (8.9 g, 23.5 mmol, 88%). The *cis*- to-*trans* ratio was determined with double resonance NMR experiments and irradiation at 2.00 ppm. Two singlets appeared at 5.34 for the *cis* and at 5.38 for the *trans* orientation. Integration of these signals gave the relative amounts of the isomers. – ¹H NMR (200 MHz, CDCl₃): δ = 0.87 (t, 3 H, CH₃), 1.26 (m, 22 H, CH₂), 1.67–1.86 (m, 2 H, CH₂CH₂I), 1.97–2.05 (m, 4 H, CH₂CH=CHCH₂), 3.19 (t, *J* = 7.1 Hz, 2 H, CH₂I), 5.32–5.40 (m, 2 H, CH).

Synthesis of Alkynyl Bromides 2i–k. – Synthesis of 1-Bromooctadec-9-yne (2k): A solution of butyllithium (2.5 M in hexane; 17 mL, 43 mmol) was added to a solution of 1-decyne (7.0 mL, 38 mmol) in THF (40 mL) cooled in an ice/salt bath. After stirring for 30 min a solution of 1,8-dibromo octane (22 mL, 110 mmol) in THF (20 mL) was added and the reaction mixture was heated under reflux for 3 h. After cooling the THF was evaporated and the residue was partitioned between water and pentane. The organic phase was dried (MgSO₄) and evaporated. The excess dibromo octane (reusable) was recovered by kugelrohr distillation (80 °C; 0.01 mbar) to leave a colorless residue (9.2 g, 28 mmol, 74%). – ¹H NMR

(200 MHz, CDCl_3): δ = 0.88 (t, 3 H, CH_3), 1.35–1.60 (m, 22 H, CH_2), 1.78–1.92 (m, 2 H, $\text{CH}_2\text{CH}_2\text{Br}$), 2.15 (m, 4 H, CH_2CCCH_2), 3.40 (t, J = 6.8 Hz, 2 H, CH_2Br). The same procedure was used to synthesize **2i** and **2j**. From 1-nonyne and 1,7-dibromoheptane, **1-bromohexadec-8-yne (2i)** was obtained in 52% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.88 (t, 3 H, CH_3), 1.27–1.60 (m, 18 H, CH_2), 1.78–1.92 (m, 2 H, $\text{CH}_2\text{CH}_2\text{Br}$), 2.15 (m, 4 H, CH_2CCCH_2), 3.40 (t, J = 6.8 Hz, 2 H, CH_2Br); from 1-octyne and 1,8-dibromooctane, **1-bromohexadec-9-yne (2j)** was obtained in 30% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.88 (t, 3 H, CH_3), 1.35–1.50 (m, 18 H, CH_2), 1.79–1.91 (m, 2 H, $\text{CH}_2\text{CH}_2\text{Br}$), 2.15 (m, 4 H, CH_2CCCH_2), 3.40 (t, J = 6.8 Hz, 2 H, CH_2Br).

General Procedure for the Synthesis of 4-Alkylpyridines: A solution of butyllithium (1.6 M in hexane; 2.05 equiv.) was added to a solution of diisopropylamine (0.2 M; 2.1 equiv.) in dry ether at -15°C . After stirring for 30 min, freshly distilled 4-methylpyridine (1 equiv.) was added dropwise. The resulting red solution was stirred for 15 min at -15°C and then a solution of alkyl halide (1 M; 2.05 equiv.) in dry ether was added in one portion. The mixture was stirred overnight at room temperature. Ether was added and the reaction mixture washed twice with 1 M NH_4Cl solution, dried with Na_2SO_4 and evaporated to dryness. The product was either crystallized from acetone or purified by chromatography on Al_2O_3 (neutral, activity II–III), gradient elution with hexane and finally hexane/ether (5:1) gave the products in 60–90%.

4-(Didodecylmethyl)pyridine (3a): White crystals from acetone (82% yield), m.p. 48°C . – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, 6 H, CH_3), 1.23 (m, 40 H, CH_2), 1.56 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.47 (m, 1 H, CH), 7.17 (d, J = 5.9 Hz, 2 H, CH_{ar}), 8.49 (d, J = 5.9 Hz, 2 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): 13.9 (p), 24.4, 26.1, 29.2, 29.3, 29.5, 31.7, 36.1 (s), 45.5 (t), 123.2 (t), 149.6 (t), 155.5 (q). – $\text{C}_{30}\text{H}_{55}\text{N}$ (429.77): calcd. C 83.84, H 12.90, N 3.26; found C 83.58, H 12.75, N 3.13.

4-(Ditetradecylmethyl)pyridine (3b): White crystals from acetone, m.p. 57°C . – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, 6 H, CH_3), 1.24 (m, 48 H, CH_2), 1.50–1.61 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.22 (m, 1 H, CH), 7.00 (d, J = 5.9 Hz, 2 H, CH_{ar}), 8.49 (d, J = 5.9 Hz, 2 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 13.8 (p), 22.4, 27.2, 29.1, 29.2, 29.4, 31.7, 36.0 (s), 45.4 (t), 123.1 (t), 149.5 (t), 155.4 (q).

4-(Dihexadecylmethyl)pyridine (3c): White crystals from acetone (90% yield), m.p. 63°C . – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, 6 H, CH_3), 1.23 (m, 56 H, CH_2), 1.56 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.47 (m, 1 H, CH), 7.17 (d, J = 6.1 Hz, 2 H, CH_{ar}), 8.49 (d, J = 6.1 Hz, 2 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 13.9 (p), 24.4, 26.1, 29.2, 29.3, 29.5, 31.7, 36.1 (s), 45.5 (t), 123.2 (t), 149.6 (t), 155.5 (q).

4-(Dioctadecylmethyl)pyridine (3d): White powder from acetone, m.p. 65°C , in 87% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, J = 6.2, 6 H, CH_3), 1.24 (m, 64 H, CH_2), 1.50 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.47 (m, 1 H, CH), 7.05 (d, J = 4.6 Hz, 2 H, CH_{ar}), 8.48 (d, J = 4.6 Hz, 2 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 13.8 (p), 22.4, 27.2, 29.1, 29.2, 29.4, 31.7, 36.0 (s), 45.4 (t), 123.1 (t), 149.5 (t), 155.4 (q).

4-(Nonadec-10-enyl)pyridine and 4-(Octadecyl, oleylmethyl)pyridine (3e): This was prepared by the initial introduction of one oleyl group from a slight excess of 4-methylpyridine. Purification by chromatography on Al_2O_3 gave a light yellow oil in 40% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, J 6.2, 3 H, CH_3), 1.27 (m, 24 H, CH_2), 1.54–1.65 [m, 2 H, $\text{CH}_2\text{CH}_2\text{Ar}$], 1.96–2.02 (m, 4 H, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 2.59 (t, J 7.6, 2 H, CH_2Ar), 5.32–5.37 (m, 2 H, CH), 7.10 (d, J = 5.4 Hz, 2 H, CH_{ar}), 8.48 (d, J = 5.1, 2 H, CH_{ar}). Treatment of this product with 1.1 equivalents of LDA and 1.05 equivalents of octadecyl bromide, followed by chromatography

on Al_2O_3 , gave **3e** as a colorless oil in 67% yield. – ^1H NMR (300 MHz, CDCl_3): δ = 0.87 (t, J 6.6, 6 H, CH_3), 1.25 (m, 56 H, CH_2), 1.45–1.65 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 1.97–2.00 (m, 4 H, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 2.43–2.49 (m, 1 H, CH), 5.31–5.81 (m, 2 H, CH), 7.05 (d, J = 5.9, 2 H, CH_{ar}), 8.48 (d, J = 5.5, 2 H, CH_{ar}). – ^{13}C NMR (75.5 MHz, CDCl_3): δ = 13.8 (p), 22.4, 26.9, 27.2, 29.1, 29.2, 29.4, 31.7, 36.0 (s), 45.4 (t), 123.1 (t), 129.7, 129.8 (t, *Ccis*), 130.2, 130.2 (t, *Ctrans*), 149.5 (t), 155.4 (q).

4-(Dioleymethyl)pyridines 3f–h: All purified by chromatography on Al_2O_3 . Compound **3f** was obtained as a colorless oil, in 73% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.89 (t, J 6.2, 6 H, CH_3), 1.27 (m, 48 H, CH_2), 1.50 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.00 (m, 8 H, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 2.43 (m, 1 H, CH), 5.34 (m, 4 H, CH), 7.06 (d, J = 4.6, 2 H, CH_{ar}), 8.49 (d, J = 4.6, 2 H, CH_{ar}). – ^{13}C NMR (75.5 MHz, CDCl_3): δ = 14.0 (p), 22.6, 27.1, 27.3, 29.1, 29.2, 29.4, 29.5, 29.6, 29.7, 31.8, 36.1 (s), 45.5 (t), 123.1 (t), 129.7, 129.8 (t, *Ccis*), 130.2 (t, *Ctrans*), 149.5 (t), 155.3 (q). Spectral data for **3g–h** were identical. Compound **3g** was obtained in 75% yield as a colorless oil. Compound **3h**, was recrystallized from acetone and obtained as white crystals, m.p. $25\text{--}26^\circ\text{C}$ in 60% yield.

4-(Dihexadec-8-ynylmethyl)pyridine (3i): Purified by chromatography on Al_2O_3 and obtained as a light yellow oil in 58% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.88 (t, J = 6.6 Hz, 6 H, CH_3), 1.20–1.73 (m, 44 H, CH_2), 2.10 (m, 8 H, CH_2CCCH_2), 2.44 (m, 1 H, CH), 7.04 (d, J = 5.9 Hz, 2 H, CH_{ar}), 8.48 (d, J = 5.5 Hz, 2 H, CH_{ar}).

4-(Dihexadec-9-ynylmethyl)pyridine (3j): Purified by chromatography on Al_2O_3 and obtained as a light yellow oil in 54% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, J = 6.6 Hz, 6 H, CH_3), 1.20–1.74 (m, 44 H, CH_2), 2.10 (m, 8 H, CH_2CCCH_2), 2.44 (m, 1 H, CH), 7.04 (d, J = 5.9 Hz, 2 H, CH_{ar}), 8.48 (d, J = 5.5 Hz, 2 H, CH_{ar}).

4-(Dioctadec-9-ynylmethyl)pyridine (3k): Purified by chromatography on Al_2O_3 and obtained as a light yellow oil in 60% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.88 (t, J = 6.6 Hz, 6 H, CH_3), 1.20–1.73 (m, 52 H, CH_2), 2.10 (m, 8 H, CH_2CCCH_2), 2.44 (m, 1 H, CH), 7.04 (d, J = 5.9 Hz, 2 H, CH_{ar}), 8.48 (d, J = 5.5 Hz, 2 H, CH_{ar}).

General Procedure for the Quaternization of 4-Alkylpyridines with Methyl Iodide: The 4-substituted alkylpyridines were quaternized with an excess (ca. 5 equiv.) of MeI (0.3 M) in acetone for 1 h at room temperature to give the corresponding 1-methylpyridinium iodide salts in quantitative yields. The iodide salts were converted into the corresponding chloride salts by ion-exchange chromatography with Dowex 1 \times 8 200–400 mesh (Cl^- form) with MeOH as eluent.

4-(Didodecylmethyl)-1-methylpyridinium Chloride (4a): White crystals from acetone. – MS (70 V); m/z : 444.8 [$\text{C}_{31}\text{H}_{53}\text{N}^+$]. – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, J = 6.5, 6 H, CH_3), 1.23 (m, 40 H, CH_2), 1.55–1.72 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.78 (m, 1 H, CH), 4.75 (s, 3 H, NCH_3), 7.71 (d, J = 6.6, 2 H, CH_{ar}), 9.45 (d, J = 6.6, 2 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 14.0 (p), 22.6, 27.3, 29.2, 29.5, 31.8, 35.7 (s), 46.5 (t), 47.9 (p), 126.9 (t), 145.3 (t), 167.0 (q).

4-(Ditetradecylmethyl)-1-methylpyridinium Chloride (4b): White crystals from acetone, m.p. $57\text{--}60^\circ\text{C}$. – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, J = 6.5, 6 H, CH_3), 1.23 (m, 48 H, CH_2), 1.55–1.72 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.78 (m, 1 H, CH), 4.75 (s, 3 H, NCH_3), 7.71 (d, J = 6.6, 2 H, CH_{ar}), 9.45 (d, J = 6.6, 2 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 14.0 (p), 22.6, 27.3, 29.2, 29.5, 31.8, 35.7 (s), 46.5 (t), 47.9 (p), 126.9 (t), 145.3 (t), 167.0 (q). – $\text{C}_{35}\text{H}_{66}\text{ClN}$ (536.37): calcd. C 78.38, H 12.40, Cl 6.61, N 2.61; found C 78.39, H 12.51, Cl 6.56, N 2.67.

4-(Dihexadecylmethyl)-1-methylpyridinium Chloride (4c): White crystals from acetone m.p. 64 °C. – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, J = 6.5, 6 H, CH_3), 1.24 (m, 56 H, CH_2), 1.48–1.78 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.81 (m, 1 H, CH), 4.75 (s, 3 H, NCH_3), 7.72 (d, J = 6.7, 2 H, CH_{ar}), 9.48 (d, J = 6.5, 2 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 13.8 (p), 22.4, 27.2, 29.1, 29.4, 31.7, 35.4 (s), 46.4 (t), 48.4 (p), 126.9 (t), 144.9 (t), 167.3 (q). – $\text{C}_{39}\text{H}_{74}\text{ClN} \cdot \text{H}_2\text{O}$ (610.49): calcd. C 76.73, H 12.55, Cl 5.81; found C 76.53, H 12.42, Cl 5.78.

4-(Diocadecylmethyl)-1-methylpyridinium Chloride (4d): An off-white powder from acetone. – ^1H NMR (300 MHz, CDCl_3): δ = 0.87 (t, J = 6.5, 6 H, CH_3), 1.24 (m, 64 H, CH_2), 1.48–1.78 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.78 (m, 1 H, CH), 4.74 (s, 3 H, NCH_3), 7.72 (d, J = 6.8, 2 H, CH_{ar}), 9.43 (d, J = 6.4, 2 H, CH_{ar}). – ^{13}C NMR (75.5 MHz, CDCl_3): δ = 13.8 (p), 22.4, 27.2, 29.1, 29.3, 29.4, 31.7, 35.4 (s), 46.4 (t), 48.4 (p), 126.9 (t), 144.9 (t), 167.3 (q). – $\text{C}_{43}\text{H}_{82}\text{ClN} \cdot \text{H}_2\text{O}$ (666.60): calcd. C 77.48, H 12.70, N 2.10; found: C 76.88, H 12.84, N 2.11.

4-(Olelyl, octadecylmethyl)-1-methylpyridinium Chloride (4e): A white solid from acetone/acetonitrile (10:1), m.p. 59–61 °C. – ^1H NMR (300 MHz, CDCl_3): δ = 0.86 (t, J = 6.5, 6 H, CH_3), 1.24 (m, 56 H, CH_2), 1.40–1.80 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 1.93–2.06 (m, 4 H, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 2.74–2.83 (m, 1 H, CH), 4.70 (s, 3 H, NCH_3), 5.31–5.38 (m, 2 H, CH), 7.75 (d, J = 6.6, 2 H, CH_{ar}), 9.26 (d, J = 6.6, 2 H, CH_{ar}). – ^{13}C NMR (75.5 MHz, CDCl_3): δ = 13.8 (p), 22.4, 26.4, 26.9, 27.1, 28.7, 28.9, 29.1, 29.2, 29.4, 31.6, 35.4 (s), 46.2 (t), 47.8 (p), 126.8 (t), 129.5, 129.7 (t, *Ccis*), 130.1, 130.3 (t, *Ctrans*), 145.3 (t), 166.6 (q). – $\text{C}_{43}\text{H}_{80}\text{ClN} \cdot \text{H}_2\text{O}$ (666.60): calcd. C 77.71, H 12.44, N 2.11; found C 76.98, H 12.60, N 2.10.

4-(Diolelylmethyl)-1-methylpyridinium Chlorides 4f–g and 4-(Dielaidylmethyl)-1-methylpyridinium Chloride 4h: These were recrystallized from acetone/acetonitrile (10:1) and obtained as white solids. The ratio of *cis* to *trans* double bonds in the products was determined by double resonance NMR experiments. Irradiation of the protons adjacent to the double bond at 2.00 ppm resulted in two singlets for the olefinic protons at 5.34 ppm for the *cis* and at 5.38 for the *trans* orientation. – MS (70 V); m/z : 608 [$\text{C}_{43}\text{H}_{78}\text{N}^+$]. – ^1H NMR (300 MHz, CDCl_3): δ = 0.87 (t, J = 6.5, 6 H, CH_3), 1.26 (m, 44 H, CH_2), 1.53 (m, 4 H, CH_2CH_3), 1.73 [m, 4 H, $\text{CH}(\text{CH}_2)_2$], 2.00 (m, 8 H, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 2.77 (m, 1 H, CH), 4.76 (s, 3 H, NCH_3), 5.35–5.37 (m, 4 H, CH), 7.72 (d, J = 6.2, 2 H, CH_{ar}), 9.50 (d, J = 6.2, 2 H, CH_{ar}). – ^{13}C NMR (75.5 MHz, CDCl_3): δ = 13.9 (p), 22.4, 26.9, 27.2, 28.9, 29.1, 29.3, 29.4, 29.5, 31.6, 35.4 (s), 46.4 (t), 48.3 (p), 126.8 (t), 129.5, 129.7 (t, *Ccis*), 130.1, 130.3 (t, *Ctrans*), 144.9 (t), 167.1 (q). – $\text{C}_{43}\text{H}_{78}\text{ClN}$ (644.55): calcd. C 80.13, H 12.20, Cl 5.50, N 2.17; found for **4f**: C 79.77, H 12.00, Cl 5.45, N 2.24; found for **4g**: C 79.53, H 12.18, Cl 5.42, N 2.24; found for **4h**: C 79.97, H 12.21, Cl 5.40, N 2.17.

4-(Dihexadec-8-ynylmethyl)-1-methylpyridinium Chloride (4i) and 4-(Dihexadec-9-ynylmethyl)-1-methylpyridinium Chloride (4j): These compounds were obtained as colorless oils. Compound **4i**: MS (70 V); m/z : 548.5 [$\text{C}_{39}\text{H}_{66}\text{N}^+$]. – **4j**: MS (70 V); m/z : 548.5 [$\text{C}_{39}\text{H}_{66}\text{N}^+$]. – ^1H NMR (200 MHz, CDCl_3): δ = 0.86 (t, J = 6.5, 6 H, CH_3), 1.20–1.74 (m, 44 H, H_2), 2.10 (t, 8 H, CH_2CCCH_2), 2.74 (m, 1 H, CH), 4.70 (s, 3 H, NCH_3), 7.70 (d, J = 6.6, 2 H, CH_{ar}), 9.50 (d, J = 6.6, 2 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 13.8 (p), 18.5, 22.4, 27.1, 28.5, 28.6, 28.8, 29.0, 29.2, 31.6, 35.5 (s), 46.4 (t), 47.7 (p), 79.9 (q), 80.1 (q), 126.8 (t), 145.4 (t), 166.8 (q).

4-(Diocadec-9-ynylmethyl)-1-methylpyridinium Chloride (4k): Obtained as a colorless oil. – MS (70 V); m/z : 604.4 [$\text{C}_{43}\text{H}_{74}\text{N}^+$]. – ^1H NMR (200 MHz, CDCl_3): δ = 0.86 (t, J = 6.5, 6 H, CH_3), 1.20–1.73 (m, 52 H, CH_2), 2.10 (t, 8 H, CH_2CCCH_2), 2.80 (m, 1 H, CH),

4.69 (s, 3 H, NCH_3), 7.70 (d, J = 6.6, 2 H, CH_{ar}), 9.50 (d, J = 6.6, 2 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 13.8 (p), 18.5, 22.4, 27.1, 28.5, 28.6, 28.8, 28.9, 29.2, 31.6, 35.5 (s), 46.4 (t), 47.7 (p), 79.9 (q), 80.1 (q), 126.8 (t), 145.4 (t), 166.8 (q).

Dialkyl Carbinols 5a–b: These were synthesized according to the literature procedure.^[5] Dihexadecyl carbinol **5a** (78% yield) was obtained as a white powder from CHCl_3 , m.p. 90–92 °C. – ^1H NMR (200 MHz, CDCl_3): δ = 0.90 (t, 6 H, CH_3), 1.30 (m, 60 H, CH_2), 3.60 (m, 1 H, CHOH). For the synthesis of diolelyl carbinol **5b**, oleyl bromide was synthesized from oleyl mesylate (see the preparation of **2f**). A mixture of oleyl mesylate (15 g, 43.3 mmol) and LiBr (12 g, 138 mmol) in acetone (500 mL) was refluxed for 3 h. After cooling, the resulting suspension was filtered and the filtrate evaporated. Ether (250 mL) was added and the precipitate removed by filtration. The filtrate was evaporated and the resulting crude product purified by chromatography on Al_2O_3 with hexane as eluent. The product was a colorless viscous oil (11.7 g, 35.3 mmol, 81%). – ^1H NMR (200 MHz, CDCl_3): δ = 0.87 (t, 3 H, CH_3), 1.26 (m, 22 H, CH_2), 1.78–1.92 (m, 2 H, $\text{CH}_2\text{CH}_2\text{Br}$), 1.97–2.05 (m, 4 H, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 3.40 (t, J = 6.8 Hz, 2 H, CH_2Br), 5.32–5.40 (m, 2 H, CH). Diolelyl carbinol **5b** was purified by chromatography on Al_2O_3 . Gradient elution with $\text{CHCl}_3/\text{Et}_2\text{O}$ gave a colorless oil (91% yield). – ^1H NMR (200 MHz, CDCl_3): δ = 0.90 (t, 6 H, CH_3), 1.3 (m, 52 H, CH_2), 2.05 (m, 8 H, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 3.60 (m, 1 H, CHOH), 5.32–5.40 (m, 4 H, CH).

Bis(alkyl)methyl Mesylates 6a–b: Mesylation was carried out as described for the preparation of **2f**. Bis(hexadecyl)methyl mesylate **6a** was obtained as a white powder from methanol (66% yield), m.p. 51–53 °C. – ^1H NMR (200 MHz, CDCl_3): δ = 0.90 (t, 6 H, CH_3), 1.30 (m, 56 H, CH_2), 1.6 [m, 4 H, $(\text{CH}_2)_2\text{CHOMs}$], 3.0 (s, 3 H, OM), 4.7 (m, 1 H, CHOMs). Bis(oleyl)methyl mesylate **6b** was obtained as a light yellow oil in 82% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.90 (t, 6 H, CH_3), 1.3 (m, 48 H, CH_2), 1.6 [m, 4 H, $(\text{CH}_2)_2\text{CHOMs}$], 2.0 (m, 8 H, $\text{CH}_2\text{CH}=\text{CHCH}_2$), 3.0 (s, 3 H, OM), 4.7 (m, 1 H, CHOMs), 5.35 (m, 4 H, CH).

General Procedure for the Preparation of 1-Alkylpyridinium Salts 7a–d: A stirred solution of the mesylates **6a–b**, approximately 10 m, in either pyridine or 4-methyl pyridine was kept at 85 °C for 24 h. Then the excess pyridine was evaporated and the product chromatographed on Al_2O_3 . Gradient elution with $\text{CHCl}_3/\text{MeOH}$ followed by ion-exchange chromatography with Dowex 1 \times 8 200–400 mesh (Cl^- form) with MeOH as eluent gave the chloride salts.

1-(Dihexadecylmethyl)pyridinium Chloride (7a): A yellow powder from CHCl_3 , (48% yield). – MS (70 V); m/z : 542.6 [$\text{C}_{38}\text{H}_{72}\text{N}^+$]. – ^1H NMR (300 MHz, CDCl_3): δ = 0.95 (t, 6 H, CH_3), 1.30 (m, 48 H, CH_2), 2.0 [m, 4 H, $(\text{CH}_2)_2\text{CHN}$], 4.8 (m, 1 H, CHN), 8.25 (m, 2 H, CH_{ar}), 8.50 (m, 1 H, CH_{ar}), 9.50 (m, 2 H, CH_{ar}). – $\text{C}_{38}\text{H}_{72}\text{ClN} \cdot \text{H}_2\text{O}$ (596.46): calcd. C 76.52, H 12.50, N 2.42; found C 77.62, H 12.40, N 2.60. – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 13.9 (p), 22.4, 25.6, 28.9, 29.0, 29.1, 29.2, 29.4, 31.7, 35.6 (s), 74.2 (t), 128.7 (t), 143.8 (t), 145.1 (q).

1-(Dihexadecylmethyl)-4-methylpyridinium Chloride (7b): A slightly red powder from CHCl_3 , (60% yield). – MS (70 V); m/z : 556.6 [$\text{C}_{39}\text{H}_{74}\text{N}^+$]. – ^1H NMR (300 MHz, CDCl_3): δ = 0.95 (t, 6 H, CH_3), 1.30 (m, 48 H, CH_2), 2.0 [m, 4 H, $(\text{CH}_2)_2\text{CHN}$], 2.7 (s, 3 H, CH_3Ar), 4.8 (m, 1 H, CHN), 8.0 (d, 2 H, CH_{ar}), 9.40 (d, 2 H, CH_{ar}). – $\text{C}_{39}\text{H}_{74}\text{ClN} \cdot \text{H}_2\text{O}$ (610.49): calcd. C 76.73, H 12.55, N 2.29; found C 76.52, H 12.51, N 2.34. – ^{13}C NMR (50.3 MHz, CDCl_3): δ = 13.9 (p), 21.9 (p), 22.4, 25.6, 28.9, 29.0, 29.1, 29.2, 29.4, 31.7, 35.5 (s), 73.2 (t), 129.1 (t), 142.8 (t), 159.0 (q).

1-(Diolelylmethyl)pyridinium Chloride (7c): Purified by chromatography on Al_2O_3 . Gradient elution with $\text{CHCl}_3/\text{MeOH}$ gave a yellow oil in 71% yield. – ^1H NMR (200 MHz, CDCl_3): δ = 0.95 (t, 6 H,

CH₃), 1.3 (m, 48 H, CH₂), 2.0 [m, 12 H, (CH₂)₂CHN and CH₂CH=CHCH₂], 4.8 (m, 1 H, CHN), 5.35 (m, 4 H, CH), 8.25 (m, 2 H, CH_{ar}), 8.50 (m, 1 H, CH_{ar}), 9.50 (m, 2 H, CH_{ar}). – ¹³C NMR (50.3 MHz, CDCl₃): δ = 13.9 (p), 22.4, 25.6, 26.9, 28.9, 29.1, 29.3, 29.4, 31.6, 32.3, 35.6 (s), 74.1 (t), 128.7 (t), 129.5, 129.8 (t), 143.8 (t), 145.1 (t).

1-(Dioleymethyl)-4-methylpyridinium Chloride (7d): Purified by chromatography on Al₂O₃. Gradient elution with CHCl₃/MeOH gave a red oil in 76% yield. – MS (70 V); *m/z*: 608.7 [C₄₃H₇₈N⁺]. – ¹H NMR (300 MHz, CDCl₃): δ = 0.95 (t, 6 H, CH₃), 1.30 (m, 48 H, CH₂), 2.0 [m, 12 H, (CH₂)₂CHN and CH₂CH=CHCH₂], 2.7 (s, 3 H, CH₃Ar), 4.8 (m, 1 H, CHN), 5.35 (m, 4 H, CH), 8.0 (d, 2 H, CH_{ar}), 9.40 (d, 2 H, CH_{ar}). – ¹³C NMR (50.3 MHz, CDCl₃): δ = 13.9 (p), 22.0 (p), 22.4, 25.6, 26.9, 28.9, 29.1, 29.3, 29.5, 31.7, 32.4, 35.5 (s), 73.3 (t), 129.2 (t), 129.6, 129.9 (t), 142.8 (t), 159.0 (q).

(Bromoalkyl)trimethylammonium Bromides 8a–c: Compounds **8a–c** were prepared according to a literature procedure.^[6] All the compounds were isolated as white crystalline solids; propyl compound **8a**, m.p. 209–212 °C; butyl compound **8b**, m.p. 132–133 °C; pentyl compound **8c**, m.p. 141–142 °C.

General Procedure for the Preparation of 1-Alkylpyridinium Salts 9a–d: A 0.1 M solution of ammonium bromide **8a–c** and 2 equivalents of pyridine **3c, f** in absolute ethanol was heated under reflux. The reaction was monitored with NMR and upon completion, after 3–6 days, the solvent was evaporated and the resulting product chromatographed on Al₂O₃. Excess pyridine **3** was recovered by elution with CHCl₃. The product was eluted with CHCl₃/10% MeOH. The resulting dibromide salt was converted into the dichloride salts by ion-exchange chromatography with Dowex 1×8 200–400 mesh (Cl[–] form) and elution with MeOH. Compound **9a** was recrystallized from THF and obtained as white crystals in 84% yield m.p. 170 °C (dec.). – MS (70 V); *m/z*: 321.3 [C₄₄H₈₆N₂⁺]. – ¹H NMR (300 MHz, CDCl₃): δ = 0.86 (t, 6 H, CH₃), 1.23 (m, 56 H, CH₂), 1.51–1.68 [m, 4 H, (CH₂)₂CH], 2.75 (m, 1 H, CH), 2.98 [m, 2 H, CH₂CH₂N(CH₃)₃], 3.39 [s, 9 H, N(CH₃)₃], 4.05 [t, 2 H, CH₂N(CH₃)₃], 5.13 (t, 2 H, N_{ar}CH₂), 7.70 (d, *J* = 5.8 Hz, 2 H, CH_{ar}), 9.87 (d, *J* = 5.8 Hz, 2 H, CH_{ar}). – ¹³C NMR (75.5 MHz, CDCl₃): δ = 13.9 (p), 22.5, 26.2, 27.2, 29.2, 29.3, 29.5, 31.7, 35.3 (s), 46.3 (t), 53.9 (p), 56.9 (s), 62.4 (s), 127.1 (t), 145.1 (t), 167.3 (q). Compound **9b** was recrystallized from CH₃CN and obtained as white crystals in 75% yield m.p. 170 °C (dec.). – MS (70 V); *m/z*: 328.4 [C₄₅H₈₈N₂⁺]. – ¹H NMR (300 MHz, CDCl₃): δ = 0.87 (t, 6 H, CH₃), 1.23 (m, 56 H, CH₂), 1.55–1.58 [m, 4 H, (CH₂)₂CH], 2.30 [m, 4 H, (CH₂)₂CH₂N(CH₃)₃], 2.75 (m, 1 H, CH), 3.39 [s, 9 H, N(CH₃)₃], 4.06 [t, 2 H, CH₂N(CH₃)₃], 5.14 (t, 2 H, N_{ar}CH₂), 7.69 (d, *J* = 6.5 Hz, 2 H, CH_{ar}), 9.87 (d, *J* = 6.5 Hz, 2 H, CH_{ar}). – ¹³C NMR (75.5 MHz, CDCl₃): δ = 13.9 (p), 22.5, 27.2, 28.6, 29.2, 29.4, 29.5, 29.6, 31.7, 35.1 (s), 46.1 (t), 53.2 (p), 59.7 (s), 65.3 (s), 127.0 (t), 144.9 (t), 166.5 (q).

Compound **9c** was crystallized from THF and obtained as white crystals in 90% yield m.p. 150 °C (dec.). – MS (70 V); *m/z*: 335.3 [C₄₆H₉₀N₂⁺]. – ¹H NMR (300 MHz, CDCl₃): δ = 0.87 (t, 6 H, CH₃), 1.03 (m, 2 H, CH₂), 1.23 (m, 54 H, CH₂), 1.55–1.73 [m, 6 H, (CH₂)₂CH and CH₂(CH₂)₂N(CH₃)₃], 2.14 [t, 2 H, CH₂(CH₂)₂N(CH₃)₃], 2.27 [t, 2 H, CH₂CH₂N(CH₃)₃], 2.73 (m, 1 H, CH), 3.41 [s, 9 H, N(CH₃)₃], 3.92 [t, 2 H, CH₂N(CH₃)₃], 5.09 (t, 2 H, N_{ar}CH₂), 7.69 (d, *J* = 5.8 Hz, 2 H, CH_{ar}), 9.85 (d, *J* = 5.8 Hz, 2 H, CH_{ar}). – ¹³C NMR (75.5 MHz, CDCl₃): δ = 14.0 (p), 22.4, 22.6, 27.3, 29.3, 29.4, 29.6, 31.8, 35.6 (s), 46.4 (t), 53.5 (p), 59.2 (s), 65.7 (s), 126.8 (t), 145.0 (t), 166.5 (q).

Compound **9d** was obtained as a white solid in 59% yield. – MS (70 V); *m/z*: 354 [C₄₉H₉₂N₂⁺]. – ¹H NMR (300 MHz, CDCl₃): δ = 0.81 (t, 6 H, CH₃), 1.20 (m, 48 H, CH₂), 1.50–1.62 [m, 4 H,

(CH₂)₂CH], 1.93 (m, 8 H, CH₂CH=CHCH₂), 2.15–2.25 [m, 4 H, (CH₂)₂CH₂N(CH₃)₃], 2.71 (m, 2 H, CH), 3.39 [s, 9 H, N(CH₃)₃], 3.98 [m, 2 H, CH₂N(CH₃)₃], 5.25 (m, 2 H, N_{ar}CH₂), 5.28 (m, 4 H, CH), 7.66 (d, *J* = 6.3 Hz, 2 H, CH_{ar}), 9.83 (d, *J* = 6.3 Hz, 4 H, CH_{ar}). – ¹³C NMR (75.5 MHz, CDCl₃): δ = 13.8 (p), 19.7, 22.4, 26.9, 27.2, 27.8, 29.0, 29.2, 29.4, 29.5, 31.6, 32., 32.3, 35.4 (s), 46.3 (t), 53.3 (p), 59.0 (s), 65.0 (s), 127.0 (t), 129.6 (t), 129.8 (t), 145.0 (t), 166.9 (q).

1-(4-Ammoniobutyl)-4-(dihexadecylmethyl)pyridinium Dichloride (11): A solution of pyridine **3c** (1.62 g, 3.0 mmol) and phthalimide **10** (564 mg, 2.0 mmol) in acetone (10 mL) was refluxed for 21 days. After evaporation of the solvent, the red product was purified on Al₂O₃. Elution with hexane/CH₂Cl₂ (1:1) afforded the excess pyridine **3** and subsequent elution with CH₂Cl₂/10% MeOH gave the product as a solid (1.05 g, 1.26 mmol, 63%). The resulting phthalimide (412 mg, 0.5 mmol) was heated overnight in HBr/HOAc (4 mL, 1:1 v/v). After cooling the crystalline compound obtained was filtered, washed with 50% HOAc and dried over P₂O₅. The product was recrystallized from THF/EtOH abs. (5:1). The dibromide salt was then converted into the dichloride salt **11** (69% yield) by ion-exchange chromatography with Dowex 1×8 200–400 mesh (Cl[–] form) with MeOH as eluent. – MS (70 V); *m/z*: 613.6 [C₄₂H₈₂N⁺]. – ¹H NMR (300 MHz, CDCl₃): δ = 0.87 (t, 6 H, CH₃), 1.25 (m, 56 H, CH₂), 1.56–1.69 [m, 4 H, (CH₂)₂CH], 2.14 [t, 2 H, CH₂(CH₂)₂NH₃], 2.35 (t, 2 H, CH₂CH₂NH₃), 2.75 (m, 1 H, CH), 3.30 (t, 2 H, CH₂NH₃), 5.14 (t, 2 H, N_{ar}CH₂), 7.71 (d, *J* = 6.6 Hz, 2 H, CH_{ar}), 9.64 (d, *J* = 6.6 Hz, 2 H, CH_{ar}). – ¹³C NMR (75.5 MHz, CDCl₃): δ = 13.9 (p), 22.5, 23.9, 27.2, 28.8, 29.2, 29.4, 29.6, 31.8, 35.3, 38.8 (s), 46.3 (t), 59.8 (s), 127.0 (t), 144.9 (t), 166.6 (q).

4-(Dihexadecylmethyl)-1-(4-guanidiniobutyl)pyridinium Dichloride (13): A mixture of amine **11** (775 mg, 1.0 mmol), pyrazole carboxamidine **12**^[7] (437 mg, 3.0 mmol), and diisopropylethylamine (387 mg, 3.0 mmol) in DMF (8 mL) was heated at 65 °C for 24 h. After cooling, ether was added (12 mL) and the precipitate, which was produced by vigorous stirring, was filtered off, washed with ether and dried. Ion exchange using Dowex 1×8 200–400 mesh (Cl[–] form) with MeOH as eluent followed by recrystallization from CH₃CN afforded white crystals, m.p. 125 °C (dec.) (420 mg, 0.58 mmol, 58%). – ¹H NMR (300 MHz, CDCl₃, 55 °C): δ = 0.89 (t, 6 H, CH₃), 1.27 (m, 56 H, CH₂), 1.55–1.74 [m, 4 H, (CH₂)₂CH], 1.86 (m, 2 H, CH₂CH₂NH), 2.29 [m, 2 H, CH₂(CH₂)₂NH₃], 2.80 (m, 1 H, CH), 3.58 (m, 2 H, CH₂NH), 5.06 (m, 2 H, N_{ar}CH₂), 7.41 [br. s, 4 H, NHC(NH₂)₂], 7.73 (d, *J* = 6.6 Hz, 2 H, CH_{ar}), 8.34 (br. s, 1 H, NH), 9.46 (d, *J* = 6.6 Hz, 2 H, CH_{ar}). – ¹³C NMR (75.5 MHz, CDCl₃): δ = 14.0 (p), 22.6, 27.3, 29.3, 29.4, 29.5, 29.6, 31.8, 35.4, 40.8 (s), 46.4 (t), 59.8 (s), 127.0 (t), 144.3 (t), 167.1 (q). – C₄₃H₈₄Cl₂N₄ (728.09): calcd. C 70.94, H 11.63, Cl 9.74, N 7.70; found C 70.94, H 11.66, Cl 9.60, N 7.85.

General Procedure for the Preparation of the Gemini Pyridinium Amphiphiles 14a–d:

A solution of α,ω-dibromo alkane, 0.1 M, and 3 equivalents of pyridine **3c, f** in acetone was heated under reflux until all alkyl dibromide had reacted (1–3 weeks). The solvent was evaporated and the product was purified on Al₂O₃. Excess pyridine **3** was recovered by elution with hexane/CHCl₃ (1:3), whilst the product was eluted with CHCl₃/6% MeOH. The resulting dibromide salt was converted into the dichloride salt by ion-exchange chromatography with Dowex 1×8 200–400 mesh (Cl[–] form) with MeOH as eluent. Compound **14a** was obtained as a waxy solid. – MS (70 V); *m/z*: 562.5 [C₇₉H₁₄₈N₂⁺]. – ¹H NMR (200 MHz, CDCl₃): δ = 0.87 (t, 12 H, CH₃), 1.23 (m, 104 H, CH₂), 1.34–1.56 (m, 8 H, CH₂), 2.01 [m, 8 H, (CH₂)₂CH], 2.75 (m, 2 H, CH), 3.23 (m, 2 H, NCH₂CH₂CH₂N), 5.14–5.21 (m, 4 H, NCH₂CH₂CH₂N),

7.66 (d, $J = 6.6$ Hz, 4 H, CH_{ar}), 9.79 (d, $J = 6.4$ Hz, 4 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): $\delta = 13.9$ (p), 22.4, 27.3, 29.1, 29.4, 29.5, 30.2, 31.7, 35.5 (s), 46.4 (t), 59.3 (s), 126.9 (t), 145.1 (t), 166.6 (q).

Compound **14b** was obtained as a white powder. – MS (70 V); m/z : 569.6 [$\text{C}_{80}\text{H}_{150}\text{N}_2^{++}$]. – ^1H NMR (200 MHz, CDCl_3): $\delta = 0.88$ (t, 12 H, CH_3), 1.04 (m, 4 H, CH_2), 1.25 (m, 108 H, CH_2), 1.53–1.92 [m, 8 H, $(\text{CH}_2)_2\text{CH}$], 2.50 [m, 4 H, $\text{NCH}_2(\text{CH}_2)_2\text{CH}_2\text{N}$], 2.76 (m, 2 H, CH), 5.14 [m, 4 H, $\text{NCH}_2(\text{CH}_2)_2\text{CH}_2\text{N}$], 7.69 (d, $J = 6.4$ Hz, 4 H, CH_{ar}), 9.78 (d, $J = 6.4$ Hz, 4 H, CH_{ar}). – ^{13}C NMR (75.5 MHz, CDCl_3): $\delta = 13.9$ (p), 22.5, 27.2, 28.2, 29.2, 29.3, 29.4, 29.5, 31.7, 35.6 (s), 46.4 (t), 59.4 (s), 127.0 (t), 145.0 (t), 166.9 (q).

Compound **14c** was obtained as a white powder. – ^1H NMR (200 MHz, CDCl_3): $\delta = 0.86$ (t, 12 H, CH_3), 1.23 (m, 114 H, CH_2), 1.45–1.90 [m, 8 H, $(\text{CH}_2)_2\text{CH}$], 2.32–2.42 [m, 4 H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}$], 2.76 (m, 2 H, CH), 5.00–5.09 [m, 4 H, $\text{NCH}_2(\text{CH}_2)_3\text{CH}_2\text{N}$], 7.70 (d, $J = 6.6$ Hz, 4 H, CH_{ar}), 10.11 (d, $J = 6.6$ Hz, 4 H, CH_{ar}). – ^{13}C NMR (50.3 MHz, CDCl_3): $\delta = 13.9$ (p), 22.5, 27.2, 29.2, 29.5, 30.3, 31.7, 35.5 (s), 46.3 (t), 59.5 (s), 126.9 (t), 145.2 (t), 166.5 (q).

Compound **14d** was obtained as a white solid. – MS (70 V); m/z : 622.4 [$\text{C}_{88}\text{H}_{158}\text{N}^{++}$]. – ^1H NMR (300 MHz, CDCl_3): $\delta = 0.87$ (t, 12 H, CH_3), 1.23 (m, 96 H, CH_2), 1.58–1.68 (m, 8 H, CH_2), 2.00 [m, 16 H, $\text{CH}_2\text{CH}=\text{CHCH}_2$], 2.49 [m, 4 H, $\text{NCH}_2(\text{CH}_2)_2\text{CH}_2\text{N}$], 2.76 (m, 2 H, CH), 5.11 [m, 4 H, $\text{NCH}_2(\text{CH}_2)_2\text{CH}_2\text{N}$], 5.34 (m, 8 H, CH), 7.68 (d, $J = 6.6$ Hz, 4 H, CH_{ar}), 9.73 (d, $J = 6.4$ Hz, 4 H, CH_{ar}). – ^{13}C NMR (75.5 MHz, CDCl_3): $\delta = 13.9$ (p), 22.5, 27.0, 27.2, 28.3, 29.0, 29.1, 29.3, 29.5, 31.7, 32.4, 35.5 (s), 46.4 (t), 59.5 (s), 127.0 (t), 129.7 (t), 129.8 (t), 144.9 (t), 166.8 (q).

Transfection Experiments: In this study we used a 7164 base pair plasmid containing the E. coli β -galactosidase gene under control of the cytomegalo virus immediate early gene promoter/enhancer (pCMV β Clontech, Palo Alto, CA, USA). DNA was isolated from E. coli using a Qiagen Plasmid Kit (QIAGEN® Inc, USA). The plasmid concentration was determined by measuring the absorption at 260 nm using the relation $1.0 \text{ OD} = 50 \mu\text{g/mL}$. The $\text{OD}_{260}/\text{OD}_{280}$ value was 1.95.

Cell Culture: The COS-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM® Gibco® The Netherlands) containing 7% of Fetal Calf Serum, 2 mM of L-Glutamine, 100 units/mL of penicillin and 100 mg/mL of streptomycin at 37 °C in CO_2/air (1:19).

Transfection Assay: Cells ($1.5 \cdot 10^5$ cells/well) were seeded in 12-well plates and were allowed to grow overnight. The complex of Saint/DOPE (1:1) and DNA was prepared at the charge ratio 2.5:1 (15 nmol lipid and 1 μg DNA in 1 mL of serum-free medium). After 10–15 min incubation at room temperature, 0.5 mL of the complex was added to the cells and incubated for 4 hours at 37 °C. The β -gal assay was performed 24 hours later.

β -gal Assay: The cells were washed with PBS and lysed with 250 μL of reporter lysis buffer (Promega, USA). 5 or 15 μL of lysate was assayed for β -galactosidase activity (using the CPRG substrate, Boehringer, Mannheim) and protein content (BCA protein assay).

An E. coli β -galactosidase standard was used for conversion of absorbance data to β -galactosidase units.

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